

## Original Research

## Determination of Circulating Tumor Cells by Flow Cytometry in the Bladder Cancer Patients

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## ABSTRACT

## Background

The current study aimed to detect of circulating tumor cells (CTCs) in 7.5 mL blood sample in bladder cancer patients treated with transurethral resection (TUR) or radical cystectomy (RC) and compare the pre-operative and post-operative CTC counts.

## Materials and Methods

CTCs were detected in peripheral blood of 10 bladder cancer patients with flow cytometry using biomarkers EpCAM, CK 14, 15, 16, 19 and CK 7, 8 for positive selection and CD45 for negative selection. The blood samples obtained before and after operation were studied in 5 out of 10 patients.

## Results

The CTC counts decreased significantly in patients ( $Z=2.032$ ;  $p=0.042$ ) after the operation. The pre-operative CTC counts for the patient group were found to be significantly higher than the control group. No CTCs were detected in the control group.

## Conclusion

The results are promising in terms of CTC detection in bladder cancer (Bca) by flow cytometry. Pre- and post-operative CTC counts as predictive and prognostic biomarker dramatically change in this study.

## Keywords

Bladder cancer (Bca); Circulating tumor cells (CTCs); Flow cytometry.

## INTRODUCTION

Cancer is a group of diseases described by unregulated cell growth and the invasion and spread of cells from the origin to other locations in body.<sup>1</sup>

## Bladder Cancer

Bladder cancer (BCa) is the 9<sup>th</sup> most common cancer worldwide and is observed in men is three to four times higher compared to women.<sup>2,3</sup> Deaths from BCa are mainly related to distant metastasis. Therefore, the prevention of metastatic disease remains a very important target in BCa.<sup>4</sup>

## Risk Factors

The main preventable risk factor for BCa is smoking cigarettes in both men and women.<sup>5</sup> In addition, occupational diseases (rubber, certain dyes and textiles, paint, and hairdressing supplies), a diet fried meats and an infection caused by *Schistosoma hematobium* are other risk factors for BCa.<sup>6</sup>

## Transurethral Resection and Radical Cystectomy

Non-muscle invasive disease is usually treated with transurethral resection of bladder tumor (TUR-BT) with or without intravesical therapy, depending on tumor grade and depth of invasion.<sup>7</sup> The

most effective treatment for the muscle invasive and recurrent high-grade non-muscle invasive urothelial carcinoma of the bladder (UCB) is radical cystectomy (RC) with bilateral pelvic lymphadenectomy with or without perioperative chemotherapy.<sup>8</sup>

### Properties of Bladder Cancers' Biomarkers

Most patients have superficial tumors and they can be completely removed. However, a great majority of these patients will have a recurrence by 15 years. In these cases, early diagnosis is very important and allows for better clinical outcomes. Potential bladder tumor markers can be used for earlier detection of recurrence; complementary testing to urine cytology to improve the detection rate and managing the cytoscopic evaluation of patient follow-up.<sup>7</sup>

An ideal BCa marker should have high sensitivity and specificity, be less expensive and more reliable compared to the urine cytology test.<sup>9</sup>

### Circulating Tumor Cells

Metastasis is the result of the dissemination of BCa cells spread into the blood or lymph nodes. However, it is difficult to diagnose metastatic disease before a surgical procedure.<sup>10</sup> Most cancer deaths are dependent on metastasis to distant organs due to the dissemination of circulating tumor cells (CTCs), which spread out from the primary tumor. CTCs are related to metastasis.<sup>11</sup>

One CTC per 10<sup>9</sup> blood cells was detected in patients with advanced tumors.<sup>12</sup> However, it is hard to detect CTC from the limited amount of blood sample. The most commonly used methods are composed of two steps; sample enrichment and detection. In the detection of CTCs, antigen presenting difference between normal blood and tumor cells is taken into consideration.<sup>13,14</sup>

Peripheral blood mononuclear cells (PBMC) are used to CTC enrichment depending on the density gradient. PBMC consisting of lymphocytes, monocytes, platelets and tumor cells; are separated from other cells such as erythrocytes, granulocytes and platelets by density differences.<sup>15</sup> There are 3 methods for this process; Ficoll gradient method, OncoQuick method and Lymphoprep method.<sup>6,15</sup> The OncoQuick method is a new system and the use of 15-35 mL blood sample in this system is the biggest disadvantage.<sup>15,16</sup> Blood contamination risk is less in Ficoll method.<sup>6</sup> There are only 2000 to 10000 leucocyte contaminations per milliliter.<sup>16,17</sup> Leukocyte contamination is minimized by CD45 depletion.<sup>18</sup> CTCs express epithelial antigens, therefore, specific antibodies against epithelial antigens are used to detect CTCs. EpCAM and cytokeratins are used for positive selection, CD45 for negative selection.<sup>16,18</sup>

Isolated cells are analyzed by different methods. The most well-known CTC detection method is the CellSearch (Janssen Diagnostics, LLC., Raritan, NJ, USA) system approved by the US FDA.<sup>19,20</sup> The CTC detection sensitivity of this system is 78.9% to 82%.<sup>21,22</sup> CTC detection in metastatic breast, prostate and colon

cancer with the CellSearch system is used prognostically in clinical practice and CTC cut-off values have been determined. These cut-off values are  $\geq 5$ ,  $\geq 5$  and  $\geq 3$  for breast, prostate and colon cancer in 7.5 mL of blood, respectively.<sup>13</sup>

In most epithelial cancers CTCs are detectable and make possible early evaluation for neoadjuvant chemotherapy in BCa. The presence of CTCs in patients undergoing cystectomy was pre-operatively an independent adverse prognostic factor for cancer-free survival.<sup>23</sup> Highly sensitive, real-time monitoring of CTCs can be applied with advanced *in vivo* flow cytometry using diverse detection schematics without the need to extract blood samples.<sup>11</sup>

Most studies have shown that, the presence of CTCs in patients after RC have a significantly increased risk of disease recurrence and mortality.<sup>8</sup>

In the study, we aim to show the pre-operation and post-operation enumeration of CTCs, using a conventional method. We preferred to use density-based (Ficoll) and immunomagnetic separation (CD45 negative selection) for the CTC enrichment and CTCs were detected and analysed by flow cytometry.

### MATERIALS AND METHODS

The peripheral blood samples (7.5 mL) were collected in vacutainer 10 mL tubes containing the anticoagulant EDTA from cancer patients (Department of Urology, Ankara Yildirim Beyazit University (AYBU) Ankara Atatürk Training and Research Hospital, Ankara, Turkey) and healthy volunteers. All patients signed an informed-consent form to participate in the study, which was approved by the Social and Human Sciences Ethics Committee of AYBU, Turkey (28.08.2015-96). Blood samples were stored at room temperature and assayed within three hours. Mononuclear cells were isolated by Ficoll gradient centrifugation. Then, the retained cells were coated with anti-CD45 microbead to eliminate leucocytes. Magnetic positive cell population were labelled with Anti CK 14, 15, 17, 19+, Anti CK 7,8+, Anti EpCAM+ and Anti CD45-.<sup>18</sup>

### Flow Cytometric Analysis of Peripheral Blood Mononuclear Cells (PBMC)

Fresh venous blood was taken from patients before and after surgery and with the density-gradient centrifugation method the peripheral blood mononuclear cells were isolated. Direct immunofluorescence assay was performed using monoclonal antibodies against cell surface markers. Sorting was carried out with BD FACS Aria™ III Cell Sorter.

### Statistical Analysis

The distribution of age and CTC counts was examined by Shapiro-wilk test. Age was expressed as mean  $\pm$  standard deviation as it showed normal distribution, CTC counts are expressed in median (min-max: minimum-maximum), categorical variables such as gender were expressed in number (%).

The CTC counts of the patient group before and after the operation and the CTC counts of the control group were analyzed by Mann-Whitney U-test. The difference in CTC counts according to operation of the patient group was analyzed by Wilcoxon test. Statistical significance level was accepted as  $p < 0.05$ .

IBM SPSS Statistics 21.0 (IBM Corp. released 2012. IBM SPSS Statistics for Windows, version 21.0, Armonk, NY, USA: IBM Corp.) program was used for statistical analysis.

## RESULTS

The patient group included one (1) female and nine (9) males. The control group contained the same number. The mean age of BCa patient and control group was calculated as  $60.70 \pm 17.07$  years and  $56.90 \pm 16.03$  years, respectively (Table 1).

All the patients in this prospective study had detectable CTCs. And no CTCs were evaluated on controls. CTCs were evaluated five non-invasive and five invasive patients. Five of ten

(50%) patients were studied before and after operation. The pre-operation and post-operation CTC counts of the patients included in our study are as in Table 2.

Median CTC count before the operation was 6.0 (min-max: 4.0 to 21.0) and after the operation was calculated as 0.0 (min-max: 0.0-5.0) (Table 3). CTC counts decreased significantly after the operation ( $Z=2.032$ ;  $p=0.042$ ). No CTC was detected in the control group. The pre-operative CTC counts of the patient group were found significantly higher than the control group ( $Z=4.047$ ,  $p < 0.001$ ). There was no statistically significant difference between the post-operative CTC counts and the CTC counts of the control group ( $p=0.254$ ).

## DISCUSSION

CTCs are primary tumor cells or metastatic cells in the circulatory system.<sup>13</sup> CTCs were first mentioned by Ashworth in 1869.<sup>24</sup> The detection of CTCs by using surface antigens is possible with modern techniques.<sup>25</sup>

**Table 1.** Demographic Characteristics.

	Patient Group	Control Group	General
Age	$60.70 \pm 17.07^1$	$56.90 \pm 16.03$	$58.80 \pm 16.23$
Gender			
Female	1 (10.0) <sup>2</sup>	1 (10.0)	2 (10.0)
Male	9 (90.0)	9 (90.0)	18 (90.0)
Invasive status	4 (40.0)	-	4 (40.0)
Operation type			
RC	2 (20.0)	-	2 (20.0)
TUR	8 (80.0)	-	8 (80.0)

<sup>1</sup>Mean  $\pm$  standard deviation

<sup>2</sup>number (%)

**Table 2.** CTC counts of patients.

Patient No	Operation type	CTC counts before opere	CTC counts after opere
1	TUR	13	0
2	TUR	8	-
3	TUR	5	-
4	TUR	5	0
5	TUR	21	-
6	TUR	6	5
7	TUR	15	-
8	RC	6	2
9	TUR	6	-
10	RC	4	0

**Table 3.** The distribution of CTC counts in patient and control group.

Patient Group	Patient Group	Control Group	Test Statistics	p
	n=10	n=10		
Pre-Operation	6.0 (4.0-21.0) <sup>2</sup>	0.0 (0.0-0.0)	4.047	<0.001
	n=5	n=10		
Post-Operation	0.0 (0.0-5.0)	0.0 (0.0-0.0)	2.070	0.254
Test Statistics	2.032	-		
p	0.042	-		

<sup>1</sup>In the control group, the results of the single measurements are given.

<sup>2</sup>median (min-max)

Today, individual therapy in cancer has become very important. With the detection and characterization of CTC in patients, this enabled us to use individual treatment. The CTC studies have reached an advanced level with the molecular characterization.<sup>26</sup> The detection methods have not been included in clinical practice as their specificity is not yet known.

In non-invasive "liquid biopsy" procedure; exosomes, free circulating DNAs and CTCs are also used in addition to mi RNAs and proteins.<sup>27</sup> CTC analyzes allow us follow-up and individualization of treatment.<sup>28</sup>

The CTC detection study with CellSearch system in patients with BCa was first performed by Naoe et al in 2007. The count was 8 (57%) of 14 BCa patients with distant metastasis, and no CTC was found in 12 BCa patients without metastasis.<sup>29</sup> In 2008, Gallagher and colleagues detected CTC with the CellSearch system in 14 (44%) of 33 patients with BCa with distant metastasis.<sup>30</sup>

There is no specified "CTC cut-off" in the CellSearch system for BCa. Up to now, the sensitivity of the CTC detection in BCa with the CellSearch system has been rather low and the detection ratio have been found 17% to 23%.<sup>23</sup> With the CellSearch system, CTC could not be detected in early stage BCa.<sup>31</sup>

CTC in BCa has been reported to be associated with progression free survival (PFS).<sup>32</sup> CTC detection have also been used in various studies, such as; with PCR-based techniques<sup>6,11</sup>, with CellSearch system<sup>31,33</sup>, but they haven't been found as sensitive as like routine urology tests.<sup>31</sup> Cytokeratins are intracellular proteins, so they can only be detected in urine after cell death.<sup>34</sup>

In this study, we used flow cytometry approach for the CTC detection. Although CTCs have a heterogeneous nature, this allows us to use multiple markers.<sup>13</sup> Various antibodies has been utilised in this method. After CD45 depletion, the detection sensitivity with flow cytometry was reported between 57% to 94%.<sup>35</sup>

However, it could not be determined a specified "CTC cut-off" for BCa by flow cytometry as the patient number was not enough.

The detection in BCa was performed using flow cytometry as in the other studies. There was a significant decrease in CTC counts for post-operation, no CTC was detected in the control group. The pre-operative CTC counts for the patient group were found to be significantly higher than the control group. The results are promising in terms of CTC detection in BCa by flow cytometry. The study showed a dramatic change between pre- and post-operation CTC counts.

There are systems which doesn't sort CTCs as mentioned in this article. In this study, we managed to count the CTCs using flow cytometry and could also perform sorting although we did not. If they cultured and characterized, this may provide a better understanding of key biological mechanisms underlying cancer

growth and dissemination.<sup>36</sup> The results obtained in this study may give opportunity to use CTC for diagnostic and therapeutic purposes.

## CONCLUSION

The results are promising in terms of CTC detection in BCa by flow cytometry. The study showed a dramatic change between pre and post-operation CTC counts as predictive and prognostic biomarker.

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## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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